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Electrophoretic Confirmation of the Species Status of *Pontia protodice* and *P. occidentalis* (Pieridae)

Arthur M. Shapiro
and
Hansjurg Geiger

Department of Zoology, University of California, Davis, California 95616

Abstract. Electrophoretic study of sympatric and allopatric populations of the taxa *Pontia protodice* and *P. occidentalis* demonstrates unequivocally that they represent closely related but independent gene pools. Each is genetically very homogeneous over its geographic range, strongly suggesting high levels of migration, colonization, and/or gene flow. *P. protodice* is less like European *P. callidice* than is Californian *occidentalis*, suggesting a possible phylogeny which agrees with previous inferences from morphology and biogeography.

Introduction

The taxa *Pontia* (or *Pieris* or *Synchloe*) *protodice* Bdv. & LeC. and *P. occidentalis* Reak. have posed an ongoing problem for Lepidopterists; though Chang (1963) demonstrated morphological differences between them and Shapiro (1976) summarized the by then copious biological and distributional information on hand—all of which tended to support their status as separate species—many workers, including some professionals, have remained unconvinced and profess to be unable to classify many specimens to one species or the other. The present study was undertaken in the hope of further clarifying their status by comparing population samples of both from areas of sympatry and allopatry, using electrophoresis to quantify genomic similarities and differences. An ancillary objective was to test the prediction that both species would show very little interpopulational differentiation, due to their apparent pattern of colonization and their epigamic behavior.

Materials and Methods

The sources of our samples are listed in Table 1. All animals were transported alive and immediately stored at -70°C until electrophoresis. Only 1984 and 1985 catches were used. All animals were determined as *protodice* or *occidentalis* by AMS, using wing phenotype, and all wings were saved for post-electrophoresis verification. Only one possibly ambiguous specimen was used in the study. The head and thorax of each

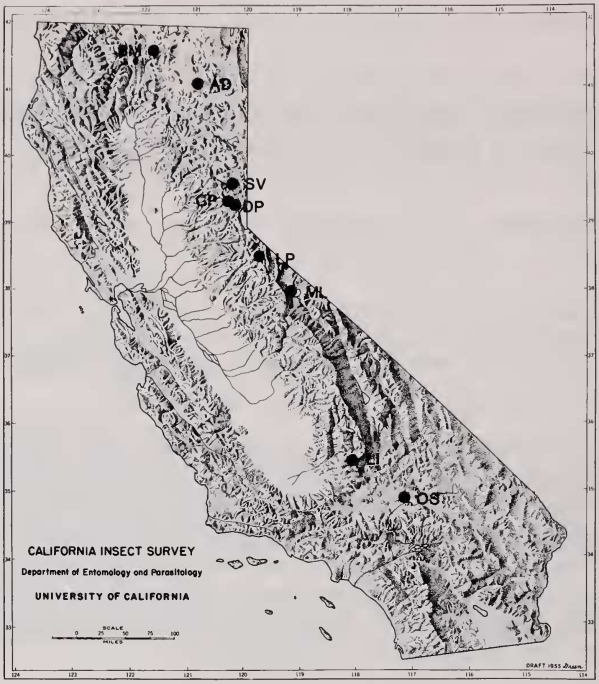

Fig. 1. Localities for California samples. Abbreviations, numbers and makeup by species as in Table 1.

butterfly were homogenized in 4 volumes of Tris-HCl buffer (0.05 M, pH 8.0). We used horizontal starch gel electrophoresis, following slightly modified standard procedures (Ayala et al., 1972; Geiger, 1981). Twenty-three enzymes were scored: acid phosphatase (locus ACPH), adenylate kinase (AK-1, AK-2), aldolase (ALD), arginine kinase (APK), fumarase (FUM), glutamate-oxaloacetate transaminase (GOT-1, GOT-2), glutamate-pyruvate transaminase (GPT), glyceraldehyde-phosphate dehydrogenase (GAPDH), α-glycerophasphate dehydrogenase (α-GPDH), hexokinase (HK), indophenol oxidase (IPO), isocitrate dehydrogenase (IDH-1, IDH-2), malate dehydrogenase (MDH-1, MDH-2), malic enzyme (ME-1, ME-2), phosphoglucomutase (PGM), 6-phospho-gluconate dehydrogenase (6-PGD), phosphoglucose isomerase (PGI), and pyruvate kinase (PK).

Analysis of the progeny of parents with different phenotypes in *Pieris brassicae* L. (Geiger, 1982) was the basis for interpreting zymograms of polymorphic loci. No deviation from the pattern observed in *P. brassicae* has been found in any individual investigated in this study. The distributions of alleles are also in good accord with Hardy-Weinberg expectations.

The most frequent allele ("common allele") in *P. brassicae* was used as a standard. This allele is designated with the index 100. Electromorphs with different mobilities are designated in relation to this standard; an allele

Table 1. Localities for samples used in this study, with notes on sympatry.

California: Lassen County: 2.5 km S Adin, 1500 m, 11.viii.1985 (n=24)(AD), *occidentalis* abundant, *protodice* unrecorded but possible infrequent immigrant. Siskiyou County: Ball Mountain, 2175 m, 10.viii.1985 (n=28)(BM), *occidentalis* only, very abundant. Sierra County: Sierra Valley, 4 km NE Sierraville, 1500 m, 25-30.vii.1985 ($n_{prot.}$=55, $n_{occ.}$=46)(SV), both abundant and permanently sympatric. Placer and Nevada Counties: Donner Pass, 2100 m, 15.viii.1985 ($n_{prot.}$=6, $n_{occ.}$=4)(DP), *occidentalis* common resident, *protodice* frequent immigrant, overwintering once in 14 yr. Nevada County: Castle Peak, 2750 m, 6.vii.1985 (n=26)(CP), *occidentalis* only (14 yrs. of observation). Alpine County: Leviathan Peak, 2800 m, 25.vii.1984 ($n_{prot.}$=2, $n_{occ.}$=17) (LP), *occidentalis* common resident, *protodice* immigrant. Mono County: nr. Mono Lake, 1800 m, 2.vii.1985 ($n_{prot.}$=17, $n_{occ.}$=2)(ML), *protodice* common, *occidentalis* infrequent (but commoner at higher elevations). Kern County: Lake Isabella, 780 m, 16.viii.1985 (n=19) (LI), *protodice* only. San Bernardino County: Route 38 N Onyx Summit, elevations not available, 15.vi.1985 (n=3)(OS), *protodice* only.

Nevada: Churchill County: vic. Fallon, 1250 m, 13.viii.1984 (n=30), *protodice* abundant, *occidentalis* very rare (none taken).

Florida: Broward County: vic. Davie, metropolitan Miami, 25.iv.1984 (n=9), *protodice* only.

Mexico: Distrito Federal: Xochimilco-San Gregorio, 27.vi-2.vii.1984 (n=12), *protodice* only.

105, then, codes for an enzyme that migrates 5 mm faster than the *P. brassicae* variant.

The statistic $\bar{I}$ (Nei, 1972) was used to estimate the genetic similarity between the samples over all loci. The calculated $\bar{I}$-values for the pooled samples of the two North American taxa plus *P. callidice* Hübner have been used to construct a dendrogram (Fig. 2) by cluster analysis (UPGMA method, Ferguson, 1980). The $\bar{I}$-values for the pooled samples are based on only 22 loci (without ACPH) to make the data comparable to an earlier study (Geiger and Scholl, 1985).

Results

At 16 of the 23 loci compared, *protodice* and *occidentalis* show only very low polymorphism ($f_{\text{common allele}} \geq 0.98$), and share the same common allele (ALD, AK-1, AK-2, APK, FUM, GADPH, GOT-2, α-GPDH, IDH-1, IDH-2, IPO, MDH-2, ME-1, ME-2, 6-PGD, PK). At four other loci (GOT-

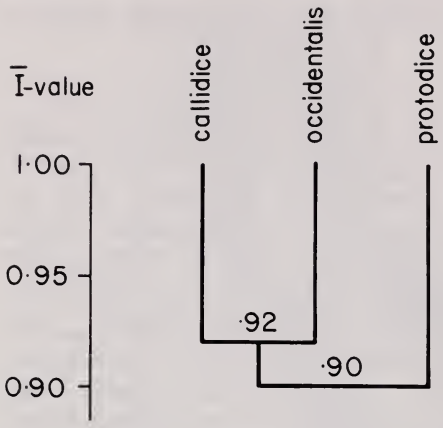

Fig. 2. Dendrogram illustrating phenetic relationship, based on Nei's I-statistic (see text.).

1, MDH-1, PGM, PGI) the two taxa have the same common allele in all samples, but are polymorphic (Table 2). For those samples with n $\geq$10, the frequencies of all alleles are remarkably similar and show no statistically significant interpopulational differences. The situation is different for the three remaining loci (ACPH, HK, GPT) (Table 3). There are two alleles at the GPT locus which are found in both taxa but at very different frequencies: the common allele in *protodice* (GPT 86) is found at very high frequency (f $\geq$0.96) in all samples of that taxon but at much lower frequencies (f $\leq$0.25) in the *occidentalis* samples. The allele 97 is the common GPT allele in *occidentalis* (f $\geq$0.75) and is very rarely recorded in *protodice* (f $\leq$0.04). The genetic differences between the taxa are even more pronounced at the HK locus, where only the allele 93 is found in *protodice* (f = 1.00); this allele occurs in *occidentalis* only at frequencies $\leq$0.02. At the ACPH locus each taxon is monomorphic for a different allele.

It is important to underscore the fact that there were *no* heterozygous individuals for the ACPH locus at those localities where the taxa are frequently to permanently sympatric (Donner, Sierra Valley—see Table 1). The one possibly ambiguous individual, a female from Sierra Valley, was electrophoretically "pure *protodice*," which taxon it most closely resembled in wing phenotype. The frequencies of heterozygotes for HK and GPT did not vary significantly between sympatric and allopatric samples.

The very similar allelic frequencies at *all* loci among population samples result in very high Ī-values for the within-taxon comparisons ($\bar{I}_{occidentalis}$ = 1.00, $\bar{I}_{protodice} \geq 0.99$) (Table 4). When the two taxa are compared the Ī-value is (as expected) lower, $x_{(\bar{I})} = 0.88 \pm 0.01$.

Because HJG had already studied European *P. callidice*, and there has been considerable speculation concerning its relationship to the American taxa (Higgins and Riley, 1970; Shapiro, 1980), we compared it to our results using the 22 loci (omitting ACPH) studied for all three taxa.

Table 2. Allelic frequencies at polymorphic loci.

		n animals	GOT-1				MDH-1		PGM					PGI				
			110	115	120	125	89	100	90	95	103	107	109	87	97	107	115	125
protodice	Sierra Valley	55		.18	.81	.02	.97	.03		.07	.90	.03		.02	.06	.84	.08	
	Lake Isabella	19		.05	.95		.84	.16	.03	.10	.84	.03		.03	.05	.89	.03	
	Mono Lake	17		.12	.85	.03	.88	.12	.03	.12	.85				.06	.91	.03	
	Donner Pass	6		.17	.67	.17	.92	.08		.08	.75	.17			.25	.75		
	Fallon	30		.17	.82	.02	1.0		.02	.08	.80	.10			.03	.92	.05	
	Florida	9		.28	.72		.95	.05			1.0					1.0		
	Mexico	12		.13	.87		.96	.04	.04	.04	.88		.04		.08	.88		.04
	all samples	153		.15	.83	.02	.95	.05	.01	.08	.86	.04	.01	.01	.06	.88	.04	.01
occidentalis	Ball Mtn	28		.07	.79	.14	1.0			.07	.75	.16	.02		.04	.93	.04	
	Adin	24	.02	.10	.71	.17	1.0			.08	.75	.15	.02		.06	.85	.08	
	Sierra Valley	46		.07	.79	.14	.97	.03	.01	.03	.76	.20		.01	.03	.90	.05	
	Castle Peak	26		.02	.81	.17	.90	.10		.02	.71	.23	.04		.04	.89	.08	
	Leviathan	17		.03	.74	.23	1.0			.09	.74	.18			.12	.82	.06	
	all samples	147	.01	.06	.77	.17	.97	.03	.01	.05	.75	.18	.01	.01	.05	.88	.06	

Table 3. Allelic frequencies at loci with high variability between the taxa.

		n animals	ACPH		GPT		HK	
			88	95	86	97	93	96
protodice	Sierra Valley	55		1.0	.99	.01	1.0	
	Lake Isabella	19		1.0	1.0		1.0	
	Mono Lake	17		1.0	.97	.03	1.0	
	Donner Pass	6		1.0	1.0		1.0	
	Fallon	30		1.0	1.0		1.0	
	Florida	9		1.0	1.0		1.0	
	Mexico	12		1.0	.96	.04	1.0	
	all samples	153		1.0	.99	.01	1.0	
occidentalis	Ball Mtn	28	1.0		.23	.77	.02	.98
	Adin	24	1.0		.23	.77	.02	.98
	Sierra Valley	46	1.0		.13	.87	.02	.98
	Castle Peak	26	1.0		.25	.75		1.0
	Leviathan	17	1.0		.06	.94		1.0
	all samples	147	1.0		.18	.82	.01	.99

Occidentalis and *callidice* cluster at a slightly higher level than *protodice*. The relationships with other species of the genus *Pontia* remain unchanged.

Discussion

We had predicted a high level of genetic similarity over large distances in these species because both are highly vagile, colonizing or "weedy" species and because *P. occidentalis* is a facultative "hilltopper," a mating strategy which would tend to promote gene flow and prevent local ecotypic differentiation. (For discussion of the population dynamics of *P. protodice*, see Shapiro, 1979; for dispersal ability of *P. protodice*, Shapiro, 1982 and of *P. occidentalis*, Shapiro, 1977; an explicit prediction was made in Shapiro, 1984, p. 181). We were nonetheless surprised at the extreme homogeneity of *protodice* over a continent-wide range (Florida, Mexico, Nevada, California). We know that these populations are not so homogeneous for such adaptive traits as the photoperiodic thresholds for induction of pupal diapause, the programming and control of diapause, hostplant adaptation and disease resistance (AMS, unpublished data). Mexican *protodice* also lay smaller eggs than other populations, even under standardized rearing conditions and on a standard diet (Shapiro, in press). All *protodice* populations tested to date have been fully reproductively compatible with one another, even in such wide crosses as New York

Table 4. I-values for the comparison of all populations samples (n >6) based on the data of 23 enzyme loci.

	protodice						occidentalis				
	Lake Isabella	Mono Lake	Donner Pass	Fallon	Florida	Mexico	Ball Mtn	Adin	Sierra Valley	Castle Peak	Leviathan
Sierra Valley	1.00	1.00	1.00	1.00	1.00	1.00	.89	.89	.88	.89	.87
Lake Isabella		1.00	1.00	1.00	1.00	1.00	.89	.88	.88	.89	.87
Mono Lake			1.00	1.00	1.00	1.00	.89	.89	.88	.89	.87
Donner Pass				1.00	.99	1.00	.88	.89	.88	.88	.87
Fallon					1.00	1.00	.89	.89	.88	.89	.87
Florida						1.00	.89	.89	.88	.88	.87
Mexico							.89	.89	.88	.89	.87
Ball Mtn								1.00	1.00	1.00	1.00
Adin									1.00	1.00	1.00
Sierra Valley										1.00	1.00
Castle Peak											1.00

X California or Texas, or Mexico X California; but the control of diapause is routinely disrupted in such wide crosses, usually resulting in failure to diapause or very rapid spontaneous termination, and much less often in extended, lethal diapause. We have less extensive experience crossing *occidentalis* populations but have found complete compatibility among California and Colorado ones and between California and the Alaskan subspecies *nelsoni* Edwards (Shapiro, 1975) which is highly incompatible with European *callidice* (Shapiro, 1980). Diapause is largely unstudied in these cases.

Vawter and Brussard (1984) found similar uniformity in the weedy, introduced species *Pieris rapae* L. in eastern North America, but more genetic diversity in the west. Populations of *P. rapae* in the west are discontinuous, separated (except in the Central Valley of California) by broad expanses of inhospitable terrain. The ability of *P. rapae*, as an obligatorily multivoltine species, to accommodate to western climates by altitudinal migration seems very limited in comparison to *P. protodice*; indeed, *rapae* is largely confined to local "mesic" pockets created by irrigation within arid or semiarid regions, while *protodice* is able to colonize throughout. This is most dramatically illustrated in central Mexico: *protodice*, a native species, is quite generally distributed, but the introduced *rapae* is ecologically "trapped" in the floating gardens of Xochimilco, near Mexico City, where continuous breeding is possible. It is hardly surprising that the homogenizing effects of gene flow are more evident in *P. protodice* than in *P. rapae.*

Vawter and Brussard argue that gene flow should be countered in colonizing or fugitive species by genetic drift and founder effect, which would tend to cause stochastic differences among populations. We are examining the genetic structure of truly ephemeral populations of *P. protodice* (presumably resulting from colonizations by single females) in the hope of addressing this question.

The clear genetic differences between *protodice* and *occidentalis* at three loci are in sharp contrast to the low variation within the taxa. The most important samples are those from Sierra Valley, where both taxa are very abundant and apparently in stable coexistence (over 10 years, AMS observations) and where occasional (<3%) ambiguous phenotypes are encountered. There is absolutely no evidence for gene flow in the sympatric Sierra Valley samples; the lack of ACPH heterozygotes shows that there were no F_1 hybrids in our collections. There may indeed be occasional, very rare spontaneous hybridization (AMS has collected one mixed pair *in copula* in Donner Pass), but the electrophoretic data provide clear confirmation that *protodice* and *occidentalis* represent separate gene pools, corresponding to biological species. There is no evidence of introgression (that is, the two taxa are not more similar genetically in sympatry than in allopatry).

It is exceedingly difficult to hybridize these two taxa spontaneously, and

pairings can normally only be secured with a pre-excited male and a substituted *teneral* allospecific female. Hand-pairings are easily achieved, but to date the level of developmental incompatibility has been high, resulting in dwarfing, high mortality, malformation, deficiency of the heterogametic sex (female), and hybrid sterility. Further information on experimental hybridization will be published elsewhere; it suffices to note that it is fully in accord with the electrophoretic results.

Genetic differences within the group of three taxa (*protodice, occidentalis, callidice*) are relatively low but within the range previously reported for closely-related species in Pieridae (Geiger, 1981; Geiger and Scholl, 1985). This observation can be interpreted as evidence for the recency of speciation in that group. Shapiro (1980) interpreted the group as derived by fragmentation of the range of a widespread circumglacial steppe-tundra entity more or less resembling the climatic adaptation of some contemporary *occidentalis* populations. The phenotypic characteristics of *protodice* are clearly derivative reductions from the full *occidentalis* pattern, which in the western United States is polyphenic and shows some reduction from the *nelsoni-callidice* pattern. Larval and pupal characters vary concordantly (Shapiro, unpublished data). The dendrogram thus further supports the proposed phylogeny, which would derive *occidentalis* from circumpolar proto-*callidice* and *protodice* in turn from *occidentalis*, without specifying time scales. Certain central Asian taxa assigned as subspecies to *callidice (orientalis* Alph., *kalora* Moore, etc.) are extremely close phenotypically to western North American *occidentalis*. This may represent parallel evolution in similar climates—but then again, it may not. Nominate *callidice* from the Alps and Pyrenees seems to represent the extreme end of a long cline, physiologically as well as geographically.

We note in closing that the extreme genetic homogeneity shown by *protodice* and *occidentalis* over a large range suggests the reality of a "general purpose genotype" associated with weediness and physiological adaptability (Baker, 1965) and the utility of these common animals as vehicles to get a closer look at its structure.

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Literature Cited

AYALA, F. J., J. R. POWELL, M. L. TRACEY, C. A. MOURAO & S. PEREZ-SALAS, 1972. Enzyme variability in the *Drosophila willistonii* group. IV. Genic variation

in natural populations of *Drosophila willistonii*. Genetics 70:113-139.

BAKER, H. G., 1965. Characteristics and mode of origin of weeds. *in* H. G. Baker and G. L. Stebbins, eds., *The Genetics of Colonizing Species*. Academic Press, N.Y., pp. 147-172.

CHANG, V. C. S., 1963. Quantitative analysis of certain wing and genitalia characters of *Pieris* in western North America. J. Res. Lepid. 2:97-125.

FERGUSON, A., 1980. *Biochemical Systematics and Evolution*. Blackie, Glasgow and London.

GEIGER, H. J., 1981. Enzyme electrophoretic studies on the genetic relationships of Pierid butterflies. I. European taxa. J. Res. Lepid. 19:181-195.

_____, 1982. Biochemisch-genetische Untersuchungen zur Systematik und Evolution von Weisslingen des europäischen Faunengebietes. Ph.D. thesis, University of Bern.

GEIGER, H. J. & A. SCHOLL, 1985. Systematics and evolution of holarctic Pierinae: an enzyme electrophoretic approach. Experientia 41:24-29.

HIGGINS, L. G. & N. D. RILEY, 1970. *A Field Guide to the Butterflies of Britain and Europe*. Houghton Mifflin, Boston. p. 50.

NEI, M., 1972. Genetic distance between populations. Am. Nat. 106:283-292.

SHAPIRO, A. M., 1975. The genetics of subspecific phenotype differences in *Pieris occidentalis* Reakirt and of variation in *P. o. nelsoni* W. H. Edwards (Pieridae). J. Res. Lepid. 14:61-83.

_____, 1976. The biological status of Nearctic taxa in the *Pieris protodice-occidentalis* group (Pieridae). J. Lep. Soc. 30:289-300.

_____, 1977. Apparent long-distance dispersal by *Pieris occidentalis* (Pieridae). J. Lep. Soc. 31:202-203.

_____, 1979. Weather and the lability of breeding populations of the checkered white, *Pieris protodice* Bdv. & LeC. (Pieridae). J. Res. Lepid. 17:1-23.

_____, 1980. Genetic incompatibility between *Pieris callidice* and *P. occidentalis nelsoni*: differentiation within a periglacial relict complex. Can. Ent. 112:463-468.

_____, 1982. A new elevational record for *Pieris protodice* in California (Lepidoptera: Pieridae). Pan-Pac. Ent. 58:162.

_____, 1984. Polyphenism, phyletic evolution, and the structure of the Pierid genome. J. Res. Lepid. 23:177-195.

_____, in press. r and K selection at various taxonomic levels in the pierine butterflies of North and South America. *in* F. Taylor and R. Karban, eds., Evolution of Insect Life Histories. Springer-Verlag, Berlin, pp. - .

VAWTER, A. T. & P. F. BRUSSARD, 1984. Allozyme variation in a colonizing species: the cabbage butterfly *Pieris rapae* (Pieridae). J. Res. Lepid. 22:204-216.